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Effects of Klotho on fibrosis and cancer: A renal focus on mechanisms and therapeutic strategies☆

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ABSTRACT

Klotho is a membrane-bound protein predominantly expressed in the kidney, where it acts as a permissive co-receptor for Fibroblast Growth Factor 23. In its shed form, Klotho exerts anti-fibrotic effects in several tissues. Klotho-deficient mice spontaneously develop fibrosis and Klotho deficiency exacerbates the disease progression in fibrotic animal models. Furthermore, Klotho overexpression or supplementation protects against fibrosis in various models of renal and cardiac fibrotic disease. These effects are mediated at least partially by the direct inhibitory effects of soluble Klotho on TGFβ1 signaling, Wnt signaling, and FGF2 signaling. Soluble Klotho, as present in the circulation, appears to be the primary mediator of anti-fibrotic effects. Similarly, through inhibition of the TGFβ1, Wnt, FGF2, and IGF1 signaling pathways, Klotho also inhibits tumorigenesis. The Klotho promoter gene is generally hypermethylated in cancer, and overexpression or supplementation of Klotho has been found to inhibit tumor growth in various animal models. This review focuses on the protective effects of soluble Klotho in inhibiting renal fibrosis and fibrosis in distant organs secondary to renal Klotho deficiency. We also discuss the structure-function relationships of Klotho domains and biological effects in the context of potential targeted treatment strategies.

Contents

Abbreviations: ACE, angiotensin converting enzyme; ADAM, a disintegrin and metalloproteinase; AKI, acute kidney injury; ANGII, angiotensin II; AT1, angiotensin II receptor type 1; AMPKα, AMP-activated protein kinase α; α-SMA, α-smooth muscle actin; CKD, chronic kidney disease; CsA, cyclosporine A; CTGF, connective tissue growth factor; DLBCL, diffuse large B cell lymphoma; DNMT1, DNA methyltransferase 1; ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; ESRD, end-stage renal disease; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FOXO1, forkhead box protein O1; HDAC, histone deacetylase; IGF1, insulin-like growth factor 1; IGFR, insulin-like growth factor receptor; IL-6, interleukin 6; IRI, ischemia/reperfusion injury; JNK, c-Jun N-terminal kinase; LRP6, low-density lipoprotein receptor-related protein 6; MME, mesangial matrix expansion; MMP, matrix metalloproteinase; Mn-SOD, manganese superoxide dismutase; mTOR, mammalian target of rapamycin; NaPi2a, sodium/phosphate co-transporter 2a; PAI-1, plasminogen activator inhibitor-1; PDGF, platelet-derived growth factor; PDFGR, platelet-derived growth factor receptor; PI3K, phospho-inositide 3-kinase; PPARγ, peroxisome proliferator-activated receptor γ; PWV, pulse wave velocity; Rac1, Ras-related C3 botulinium toxin substrate 1; RIG-1, retinoic acid-inducible gene 1; S100A4, S100 calcium-binding protein A4; SIRT1, sirtuin 1; TGFβ1, transforming growth factor β1; TGFβR, transforming growth factor β receptor; TRPC1, transient receptor potential cation channel, subfamily C, member 1; TRPC3, transient receptor potential cation channel, subfamily C, member 3; TRPC6, transient receptor potential cation channel, subfamily C, member 6; TRPV5, transient receptor potential cation channel, subfamily V, member 5; UUO, unilateral ureteral obstruction; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 1; Wnt, wingless-related integration site; WISP1, Wnt1-inducible signaling pathway protein 1.

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1. Introduction

Fibrosis can be defined as an exaggerated response to tissue damage leading to excessive deposition of extracellular matrix. This process may become maladaptive and impair organ or tissue function. Renal fibrosis, for instance, is a shared feature of chronic kidney disease (CKD) irrespective of primary etiology, providing a rationale for the development of currently lacking anti-fibrotic drugs. One promising protein uniquely poised to provide the basis for anti-fibrotic treatment strategies is the renal anti-ageing protein Klotho. Deficiency of Klotho in mice leads to a phenotype resembling human ageing, including a short lifespan, kyphosis, osteoporosis, vascular calcification, pulmonary emphysema, gonadal atrophy, and cognitive dysfunction [1]. Overexpression of Klotho, on the other hand, extends lifespan by 20–30% [2] and protects to a large extent from renal disease [3–8], cardiac disease [8–12], pulmonary damage [13,14], neurodegenerative disease [15–19], vascular disease [20–22], and diabetes [23,24].

Klotho is a membrane-bound protein primarily expressed in the kidney, mostly in the distal tubule and at a low level in the proximal tubule (see Fig. 1B), as well as in the parathyroid gland, choroid plexus, and sinoatrial node [1,25,26]. Membrane-bound Klotho is a single-pass transmembrane protein with a 10 aa intracellular domain that has not been found to have a function in signal transduction. The extracellular part of Klotho contains two homologous domains, termed KL1 and KL2, that share a high degree of sequence similarity [27–29]. Both below KL2, just above the membrane, and in between KL1 and KL2, cleavage sites are targeted by ADAM10 and ADAM17, producing soluble Klotho proteins that either contain KL1, KL2, or both [30–32] (see Fig. 1A). It appears that the predominant soluble Klotho protein is the one of 130 kDa, containing both KL1 and KL2 [33], and that further cleavage is dependent on the generation of this 130 kDa soluble Klotho protein [34], although it should be noted that neither secondary cleavage product has been detected in human serum so far, only in *in vitro* systems. Finally, an alternatively spliced Klotho mRNA transcript has been hypothesized to code for a secreted Klotho protein [27,28], which would amount to the KL1 domain with a unique 10 aa tail, but this putative protein has proven rather elusive and has not been identified.

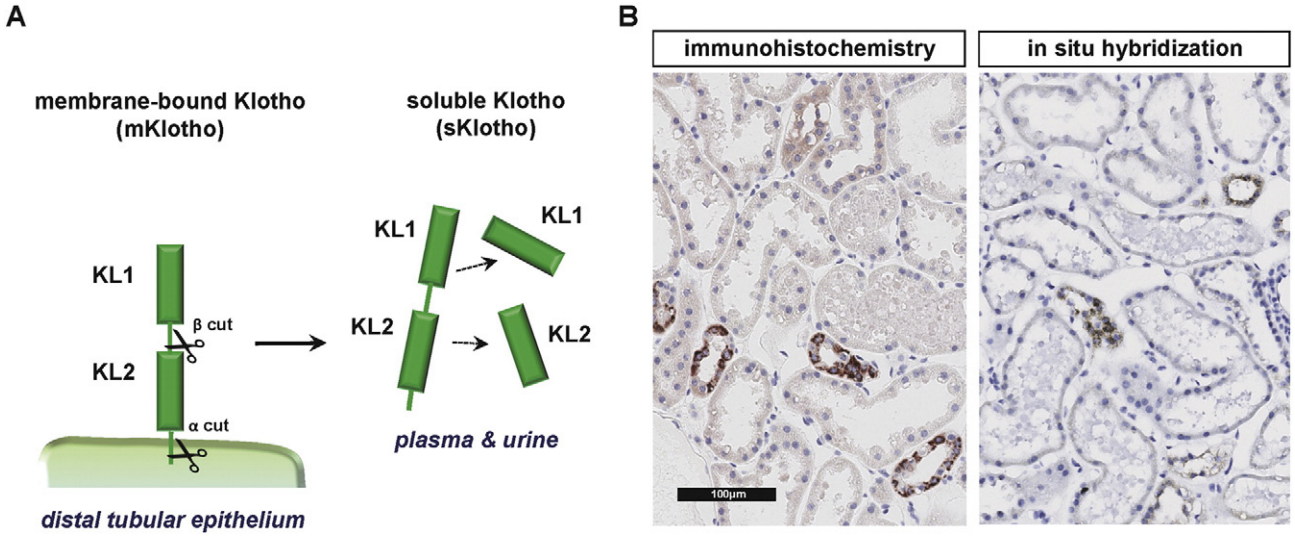

Fig. 1. Klotho protein forms and Klotho expression pattern. A) Schematic representation of Klotho proteins. Membrane-bound Klotho contains a small intracellular domain, a transmembrane domain, and a large extracellular domain consisting of two homologous domains termed KL1 and KL2. Proteolytic cleavage occurs at the indicated α cut and β cut sites, giving rise to soluble Klotho proteins, comprising the entire extracellular domain, or the single KL1 or KL2 domains. B) Immunohistochemistry and *in situ* hybridization for Klotho protein (using antibody KM2076) and Klotho mRNA, respectively, on human kidney tissue. Klotho is expressed predominantly in the distal convoluted tubule, with lower expression in the proximal tubule. No positivity is observed in tubulointerstitial cells. Original magnifications: 200×.

The anti-ageing functions ascribed to Klotho have been partially attributed to its function as a membrane-bound co-receptor for Fibroblast Growth Factor (FGF)23, promoting phosphaturia and inhibition of 1-alpha-hydroxylation of 25-hydroxyvitamin D [35,36]. However, there is mounting evidence that several of the phenotypic traits in Klotho deficient mice are mediated by soluble Klotho, which is derived from shedding of the membrane-bound protein and can be found in the circulation and the urine as a humoral factor [30,33] (see Fig. 1A). Soluble Klotho effects are mediated by direct modulation of several fundamental signaling pathways, including TGFβ1 [37], Wnt [38,39], IGF1 [2,40], and FGF2 signaling [41]. It is *via* these pathways that Klotho is thought to exert its marked anti-fibrotic effects. Furthermore, as many of these pathways affect both the development of fibrosis and tumorigenesis, a number of studies have identified Klotho as a tumor suppressor gene [40,42,43]. Klotho is primarily derived from the kidney [44] and any damage to the kidney will result in depression of Klotho expression [20,45,46]. This places the kidney in a central position as a source of soluble Klotho, a protein with demonstrated anti-fibrotic effects on several distant organs. We will discuss the current state of the evidence with regard to Klotho and fibrosis, and explore the possibilities for future treatment strategies based on Klotho delivery.

2. Soluble Klotho as an anti-fibrotic agent

When Klotho deficiency was discovered to lead to a phenotype resembling human ageing [1], it was not immediately clear that Klotho would become a subject of interest in fibrosis research. Over the years, as various strategies of Klotho overexpression and supplementation had been employed in various models of fibrotic diseases, it became increasingly apparent that Klotho is an endogenous inhibitor of the pathological fibrotic response. Interestingly, experiments using models such as unilateral ureteral obstruction (UUO) and ischemia-reperfusion injury (IRI) have been replicated many times, providing us with a solid scientific basis for discussion of the anti-fibrotic effects exerted by Klotho. We will herein focus first on establishing this relationship between Klotho and fibrosis, before addressing the molecular mechanisms.

2.1. Klotho and renal fibrosis

2.1.1. Klotho deficiency induces and promotes renal fibrosis in vivo

As a primarily renal protein with extensive renoprotective effects, it is perhaps surprising that Klotho deficiency only leads to a relatively mild renal phenotype. Fully Klotho-deficient mice display both vascular and tubular calcification [47], a decline in renal function [20], and a mild degree of interstitial fibrosis, as indicated by increased collagen deposition on Masson Trichrome staining, and an increase in α-smooth muscle actin (SMA) expression [48]. As expected, heterozygous Klotho mice ($Klotho^{+/-}$) have a less striking phenotype compared to full Klotho knockout mice ($Klotho^{-/-}$) mice and few pathological changes at a comparable age [49–51], but they also have a lifespan longer than the 8–10 weeks $Klotho^{-/-}$ mice generally experience. At around 16 weeks of age, $Klotho^{+/-}$ mice have been shown to develop both glomerulosclerosis and interstitial fibrosis, accompanied by albuminuria and a decline in renal function [52,53]. Interestingly, these mice also develop mesangial matrix expansion (MME), which is another typically ageing-related renal lesion characterized by increased extracellular matrix deposition by mesangial cells [49,52], as well as collapsing of glomeruli and tubular dilation, atrophy, and cast formation [52]. As a general observation, it is probably best to regard $Klotho^{-/-}$ mice as a model for the premature ageing that occurs in severe human Klotho deficiency, like end-stage renal disease (ESRD), in which the development of vascular calcification is also a dominant feature. On the other hand, the milder $Klotho^{+/-}$ phenotype appears to be more akin to partial human Klotho deficiency, like in physiological ageing or mild to moderate chronic kidney disease (CKD). As a rule, $Klotho^{-/-}$ mice are too fragile to withstand the demands of anaesthesia and surgery, which only allows us to establish that $Klotho^{+/-}$ mice display an increased susceptibility to the development of a pathological response to injury, including fibrosis, which is also characteristic of ageing. For example, after UUO in $Klotho^{+/-}$ and wild-type (WT) mice, Sugiura et al. found that $Klotho^{+/-}$ mice exhibited more renal fibrosis, as well as markedly higher expression levels of α-SMA, fibronectin, TGFβ1, and S100A4, coupled with a more pronounced loss of E-cadherin and endogenous Klotho expression [48]. Satoh et al. describe a similarly exaggerated response after UUO, cementing that a "second hit" will readily expedite fibrosis development in $Klotho^{+/-}$ mice [54], as do Sun et al. [55]. Paradoxically, however, the latter authors also report less fibrosis after UUO in hypomorphic Klotho (*kl*/*kl*) mice, which is at odds with other reports that indicate that these almost fully Klotho-deficient mice generally do not survive surgery [48,56]. Extending the discussion to other models, Shi et al. found that mice with one hypomorphic allele for Klotho (*kl*/+), develop more extensive renal fibrosis and have higher expression levels of α-SMA, CTGF, and collagen I, 20 weeks after bilateral IRI, compared to WT mice [7]. Overall, it appears that both $Klotho^{-/-}$ and $Klotho^{+/-}$ mice are prone to the development of renal fibrosis, although the complexity and timeline of the Klotho deficiency phenotype constitute a challenge.

Acquired rather than genetic Klotho deficiency is also associated with fibrosis, notwithstanding that causal relationships cannot be inferred from associations. In various models of renal fibrosis, including UUO [39,41,48,54,57–59], adriamycin nephropathy [39,60], cyclosporine A nephropathy [61–65], IRI [7,8], 5/6th nephrectomy [66], doxorubicin nephropathy [67], hypertension [22] (also with the addition of indoxyl sulfate) [68], uremic toxemia [69], renal artery constriction [70], adenine nephropathy [59,71–73], and diabetic nephropathy [74], fibrosis has been shown to develop while Klotho expression is concurrently decreased, possibly potentiating or exacerbating the development of fibrosis. More evidence supporting this notion of causality stems from *in vivo* RNA interference as a model of acquired Klotho deficiency. In both UUO- and adenine-induced renal failure models, *in vivo* Klotho siRNA treatment exacerbates the development of renal fibrosis and even potentiates the spontaneous development of fibrosis in sham or control mouse kidneys [57,59,71–73]. Similarly, in all stages of CKD but especially in ESRD patients in which we know renal fibrosis is present, we also know that Klotho expression is extremely decreased [20,45,46]. It is currently unknown whether mutations that confer an impairment of Klotho function also induce renal fibrosis [75].

Taken together, it is well-established that Klotho deficiency is both associated with renal fibrotic disease, as well as induces and exacerbates the development of renal fibrosis in many different models and in different species.

2.1.2. Klotho protects against the development of renal fibrosis in vivo

In addition to studies of fibrosis in Klotho deficiency, studies on experiments with Klotho overexpression or supplementation provide evidence from which we can gauge its possible therapeutic potential. One early indication that soluble Klotho is important in mediating the anti-fibrotic effects is a study by Chen et al., in which *kl*/*kl* mice were injected with soluble Klotho protein and developed less renal fibrosis, indicating that soluble Klotho protein can ameliorate fibrosis induced by Klotho deficiency [76].

More systematically, though, multiple UUO experiments supply evidence that various strategies are successful in attenuating the development of renal fibrosis. First of all, constitutive Klotho overexpression in transgenic mice was shown to inhibit fibrosis, as well as collagen III, CTGF, TGFβ1, fibronectin, cMyc, WISP1, β-catenin mRNA expression levels, in addition to attenuating the decline in renal mass [54]. Fibronectin protein levels, β-galactosidase activity, activated Rac1 levels, and phosphorylated JNK levels were also decreased compared to WT mice. Interestingly, a similar effect was achieved by overexpressing Klotho in $Klotho^{+/-}$ mice that underwent UUO using skeletal muscle electroporation, resulting in less fibrosis than in WT mice, also

demonstrative of its potential [54]. The finding that ectopically overexpressed Klotho protein can prevent the development of renal fibrosis suggests that a humoral factor, like soluble Klotho, is responsible for the anti-fibrotic effects. Also illustrative of the therapeutic anti-fibrotic potential is the finding that induction of Klotho overexpression both at 1 day before and even 3 days after UUO resulted in a marked reduction of fibrosis (including fibronectin and α-SMA protein expression), demonstrating how Klotho is capable of preventing fibrosis even after the damage response has started to develop [39]. The hypothesis that these anti-fibrotic effects are mediated by soluble Klotho is further substantiated by three studies in which UUO was performed in mice that were then treated with various concentrations of recombinant Klotho protein [37,41,77]. Assessing the effects of soluble Klotho on fibrosis-related gene and protein expression after UUO, Doi et al. found that Klotho dose-dependently decreased α-SMA and collagen I mRNA and protein levels, as well as vimentin, Snail, Twist, MMP-2, MMP-3, and MMP-9 mRNA levels, whereas, interestingly, TGFβ1 mRNA levels were unaffected [37]. Similarly, Wu et al. found that mRNA levels of α-SMA, collagen I, CTGF, MMP-2, and vimentin were decreased by Klotho, without an effect on TGFβ1 mRNA [77]. Perhaps the effects of partial Klotho deficiency and increased Klotho levels on TGFβ1 are different, but this is currently not clear. In the study by Guan et al., it was found that protein levels of FGF2, fibronectin, and α-SMA were decreased by soluble Klotho treatment, while E-cadherin protein expression was preserved [41]. Finally, one UUO study in rats indicates that Klotho protein treatment may have similar effects also in other species [78].

Another line of evidence that has started to explore the therapeutic potential for Klotho as a treatment for renal fibrosis, is a series of studies on IRI (in which Klotho overexpression had already been shown to be protective [3]). Bilateral IRI in mice that constitutively overexpress Klotho lead to less fibrosis and expression of fibrosis-related proteins, like α-SMA, collagen I, and CTGF [7], compared to in WT mice. Interestingly, similar effects were found at 2, 4, and even 20 weeks after AKI if mice were treated with soluble Klotho for only 4 days after induction of bilateral IRI, attesting to the therapeutic potency of a hypothetical Klotho-based treatment, even after the induction of renal damage [7,8]. Another example of this is the finding that starting soluble Klotho treatment 4 weeks after the induction of CKD (uninephrectomy + 30 min of contralateral IRI + high phosphate diet) and continuing Klotho treatment for 3 months in these mice with established CKD, renal fibrosis was reduced in both the Klotho-treated CKD mice and Klotho-treated sham mice (that had received a high phosphate diet), compared to vehicle-treated controls [8]. These studies also indicate that to prevent renal fibrosis, treatment with Klotho protein is potentially beneficial even if it is not administered before or directly after the occurrence of a renal ischemic insult.

In addition to UUO and IRI, other renal disease models have been used as well to test the effects of overexpression or supplementation of Klotho on renal fibrosis. For instance, Klotho gene delivery markedly reduced fibrosis in adriamycin nephropathy in mice, coupled with lower expression levels of β-catenin, Snail1, PAI-1, and fibronectin [39]. Klotho protein treatment in adriamycin nephropathy completely prevented the development of renal fibrosis as well as up-regulation of fibronectin and loss of E-cadherin on the mRNA level [60]. In another model, Klotho gene delivery prevented the development of fibrosis induced by 5/6th nephrectomy after 6 weeks in mice [66] and, extending the discussion to other species as well, in diabetic streptozotocin-injected rats Klotho gene delivery also prevented renal fibrosis, as well as fibronectin and vimentin protein expression [74]. In 24-week-old spontaneously hypertensive rats (SHR), Klotho gene delivery at 12 weeks of age also prevented the development on hypertension-induced renal fibrosis [22]. Finally, Klotho gene delivery has been shown to reduce cyclosporine A (CsA) nephropathy-induced renal fibrosis both in rats, including reduced α-SMA and TGFβ1 and increased E-cadherin mRNA and protein levels [61], and in mice [62].

A different approach that has been studied is to increase endogenous Klotho levels in order to decrease renal fibrosis. This is most commonly accomplished by employing strategies targeting epigenetic regulation of gene expression. This approach, however, does not allow for differentiation between the effects of different Klotho proteins. Sun et al. were the first to show that the Klotho promoter is hypermethylated by uremic toxins, which concurrently resulted in renal fibrosis [69]. Using 5-Aza-2dc as a DNA methyltransferase 1 (DNMT1) inhibitor, Klotho expression was increased *in vivo*, but the effect on fibrosis was not reported. Yin et al., however, recently performed a similar experiment and found that inhibition of DNMT1 both increased Klotho expression and decreased renal fibrosis in UUO. This attenuation was abrogated by Klotho siRNAs, indicating that Klotho rather than any other DNMT1-demethylated gene is essential in preventing renal fibrosis [59]. The same group reported similar results after using a different compound, rhein, which also demethylated the Klotho promoter, resulting in less fibrosis (and more fibrosis after RNA interference for Klotho) in both UUO and adenine-induced renal failure models [57,71]. A similar approach has been tried successfully with a histone deacetylating agent that also increase Klotho expression, showing that in adenine-induced CKD, HDAC inhibitor trichostatin A both decreased renal fibrosis and increased Klotho expression, an effect that was abolished in the presence of Klotho siRNAs, illustrating the key role Klotho plays in this process [72]. More specifically, HDAC3 inhibitor RGFP966 de-repressed Klotho expression *via* PPARγ and prevented renal fibrosis, but only in the absence of Klotho siRNAs [73]. Klotho expression was also increased by inhibition of H3K9 methyltransferase G9a either pharmacologically using BIX01294 or after RNA interference, coincident with less α-SMA, fibronectin, and collagen-I protein expression [58]. Other strategies that have shown that up-regulation of Klotho coincides with a halted development of fibrosis include losartan treatment [79], pravastatin treatment [64], *N*-acetylcysteine treatment [63], and curcumin treatment [65], all in cyclosporine A nephropathy, as well as aliskiren treatment in chronic ischemic kidney injury *via* renal artery constriction [70] and TGFβRI inhibitor SB431542 treatment in adenine nephropathy [73]. No causality, however, between an up-regulation or a retention of Klotho expression and the outcome of a reduction in renal fibrosis can be inferred from these studies.

To summarize, reports indicate that soluble Klotho, either directly supplemented as recombinant protein or derived from induced or constitutive overexpression, is capable of inhibiting the development of renal fibrosis in various models and multiple species. Details from the studies in which Klotho overexpression or supplementation has been used in models of renal fibrosis, are summarized in Table 1.

2.2. Klotho and cardiac fibrosis

2.2.1. Klotho deficiency induces and promotes cardiac fibrosis in vivo

The effects of Klotho on fibrosis in the heart have been the subject of a number of studies. As Klotho is not expressed in the heart except for in the sinoatrial node [25], most effects Klotho exerts on the heart are expected to be mediated by kidney-derived soluble Klotho present in the circulation [44]. Klotho deficiency leads to sinoatrial node dysfunction and consequently to arrhythmias [25], as well as to an increase in heart weight/body weight according to some [12,80], but not all accounts [10,11]. Left ventricular ejection fraction, stroke volume, and cardiac output were all found to be reduced in *kl*/+ mice compared to WT mice [9]. With regard to cardiac fibrosis, reports differ a bit: Hu et al. describe that *kl*/*kl* mice have more spontaneous cardiac fibrosis at 6 weeks, and even more at 12 weeks of age, coupled with more collagen-I and α-actinin and β-myosin heavy chain expression [9]. Heterozygotes were not found to have more spontaneous fibrosis at these ages. Xie et al., however, did not find more spontaneous cardiac fibrosis in *kl*/+ mice [11]or in *kl*/*kl* mice [10]. This discrepancy with the study by Hu et al. may be due to the use of a low-phosphate diet that increases Klotho expression and generally improves the phenotype of *kl*/*kl* mice [81,82].

Table 1
Studies using Klotho treatment in animal models of renal fibrosis.

Klotho intervention	Treatment regimen	Fibrosis model	Treatment duration/time points	Recombinant Klotho source	Severity of model	Effect on fibrosis	Effect size on fibrosis	Reference
Klotho protein treatment (i.p.)	20 μg/kg/48 h	*Kl*/*kl*mice	3–8 weeks of age	Self–made, rat Klotho	Mild	Decreased	Moderate	[76]
Genetic Klotho over expression		UUO in Klotho–Tg mice	Day 3, 7, 14		Severe	Decreased	Large	[54]
Induced Klotho over expression		UUO in *kl*/ + mice	Day 14		Severe	Decreased	Large	[54]
Induced Klotho over expression	1 day before UUO	UUO in mice	Day 7		Severe	Decreased	Large	[39]
Induced Klotho over expression	3 days after UUO	UUO in mice	Day 7		Severe	Decreased	Large	[39]
Klotho protein treatment (i.p.)	10 or 20 μg/kg/48 h	UUO in mice	Day 3, 7	Self–made, rat Klotho	Severe	Decreased	Large	[37]
Klotho protein treatment (i.p.)	10 μg/kg/48 h	UUO in mice	Day 3, 7, 14	R&D Systems, mouse Klotho	Severe	Decreased	Large	[41]
Klotho protein treatment (i.p.)	10 μg/kg/48 h	UUO in mice	Day 7	R&D Systems, human Klotho	Severe	Decreased	Large	[77]
Klotho protein injection (i.p.)	20 μg/kg/48 h	UUO in rats	Day 14	?, rat Klotho	Severe	Decreased	Large	[78]
Genetic Klotho over expression		Bilateral IRI in mice	20 weeks	Self–made, mouse Klotho	Moderate	Decreased	Large	[7]
Klotho protein treatment (i.p.)	10 μg/kg for 4 days	Bilateral IRI in mice	2, 4, 20 weeks	Self–made, mouse Klotho	Severe	Decreased	Large	[7, 8]
Klotho protein treatment (mini–pump, i.p.)	300 μg/kg/month	Uninephrectomy + IRI + HPD	3 months	Self–made, mouse Klotho	Severe	Decreased	Large	[8]
Induced Klotho over expression		Adriamycin nephropathy	3 weeks		Severe	Decreased	Large	[39]
Induced Klotho over expression		5/6th nephrectomy	6 weeks		Moderate	Decreased	Large	[66]
Induced Klotho over expression		STZ–induced diabetic nephropathy	12 weeks		Mild	Decreased	Moderate	[74]
Induced Klotho over expression		Hypertension in SHR	12 weeks		Moderate	Decreased	Large	[22]
Klotho protein injection (i.p.)	10 μg/kg/48 h (?)	CsA nephropathy in rats	4 weeks	R&D Systems, mouse Klotho	Severe	Decreased	Large	[61]
Induced Klotho over expression		CsAnephropathy in mice	4 weeks		Moderate	Decreased	Moderate	[62]

UUO, unilateral ureteral obstruction; IRI, ischemia-reperfusion injury; HPD, high-phosphate diet; STZ, streptozotocin; CsA, cyclosporin A; SHR, spontaneously hypertensive rats.

Conversely, this is substantiated by the finding that a high-phosphate diet does spontaneously induce cardiac fibrosis in *kl*/+ mice at later ages (9 months and 15 months) [9]. Furthermore, ageing itself substantially exacerbated the development of cardiac fibrosis in these mice. Xie et al. as well, using a "second hit", do describe a marked increase in cardiac fibrosis in *kl*/*kl* mice compared to WT littermates after administration of isoproterenol, a model for stress-induced cardiac hypertrophy [10]. In *kl*/+ mice as well, it was found that cardiac fibrosis secondary to 5/6th nephrectomy was dramatically increased compared to WT littermates [11], indicating an increased susceptibility to the induction of cardiac fibrosis. It should be noted that 5/6th nephrectomy in *kl*/+ mice also exacerbated their Klotho deficiency. Taken together, these findings indicate that complete Klotho deficiency may be accompanied by a mild degree of cardiac fibrosis and that partial Klotho deficiency leads to an increased propensity to developing cardiac fibrosis upon a "second hit".

2.2.2. Klotho protects against the development of cardiac fibrosis in vivo

Analogous to the kidney, different approaches and different models have been employed to assess the effects of Klotho on cardiac fibrosis. Hu et al. show that in Klotho-overexpressing mice fed a high-phosphate diet until the age of 9 months and until the age of 15 months, there was less cardiac fibrosis than in WT littermates [9]. Only one study, by Xie et al., has examined whether induction of Klotho expression can inhibit the development of cardiac fibrosis. They used *kl*/+ mice and induced cardiac fibrosis by 5/6th nephrectomy. Klotho gene delivery resulted

Table 2
Studies using Klotho treatment in animal models of cardiac fibrosis.

Klotho intervention	Treatment regimen	Fibrosis model	Time points	Recombinant Klotho source	Severity of model	Effect on fibrosis	Effect size on fibrosis	Reference
Genetic Klotho over expression		5/6th nephrectomy	4 weeks		Severe	Decreased	Large	[11]
Genetic Klotho over expression		HPD in ageing mice	9, 15 months		Moderate	Decreased	Mild	[9]
Genetic Klotho over expression		Angiotensin II in mice	4 weeks		Very mild	Increased	Small	[83]
Klotho protein treatment (i.p.)	10 μg/kg/48 h	Isoproterenol in mice	Day 2, 5, 9	R&D Systems, mouse Klotho	Moderate	Decreased	Large	[84]
Klotho protein treatment (i.p.)	10 μg/kg for 4 days	Bilateral renal IRI in mice	20 weeks	Self–made, mouse Klotho	Moderate	Decreased	Large	[8]
Klotho protein treatment (mini–pump, i.p.)	300 μg/kg/month	Uninephrectomy + renal IRI + HPD	3 months	Self–made, mouse Klotho	Severe	Decreased	Large	[8]

HPD, high-phosphate diet; IRI, ischemia-reperfusion injury.

in circulating Klotho levels still well below normal WT Klotho levels, but the development of cardiac fibrosis 35 days after 5/6th nephrectomy was markedly lower than in vector-treated *kl*/+ mice [11]. Surprisingly, one study, on angiotensin II infusion in Klotho-overexpressing mice, describes that there was actually a bit more fibrosis in these mice, for unclear reasons [83]. More experiments, however, have been performed to investigate the effects of soluble Klotho protein on cardiac fibrosis. Song et al. treated mice with isoproterenol and Klotho and found that cardiac fibrosis, both within the myocardium and associated with intramyocardial arteries, was decreased at days 5 and 9, compared to isoproterenol-treated mice [84]. Collagen I and III mRNA levels were also decreased by Klotho treatment at 9 days after the start of isoproterenol treatment [85]. Hu et al. used Klotho treatment in models of AKI-induced cardiomyopathy and CKD-related cardiomyopathy [8]. After bilateral IRI, mice were treated with Klotho for 4 days and cardiac fibrosis was found to be much less extensive 20 weeks after surgery. Protein levels of α-actinin and α-SMA were also decreased. It should be noted that progression from AKI to CKD was prevented in these mice, so it is not immediately clear whether the inhibition of fibrosis is the direct result from Klotho protein effects exerted on the heart, or whether the prevention of cardiac fibrosis secondary to CKD is prevented by Klotho effects on the kidney. In their CKD study, using uninephrectomy, contralateral IRI, and a high-phosphate diet, Klotho treatment was started 4 weeks after surgery for 12 subsequent weeks. In these mice as well, Klotho markedly inhibited the development of cardiac fibrosis, as well as lowered the protein levels of α-actinin and α-SMA. As this experiment again begs the question whether the heart is protected directly from developing fibrosis, or is protected by the prevention of renal disease-induced fibrosis, the points should be made that Klotho itself is at least one of the kidney-derived factors that may prevent cardiac fibrosis and that Klotho also protects against cardiac fibrosis in the isoproterenol model, which is not dependent on renal injury. Finally, as uremic toxins like indoxyl sulfate, the accumulation of which is the result of renal disease, are known to induce cardiac fibrosis, in part due to down-regulation of Klotho [69], many of the effects that those uremic toxins exert on the heart are also found to be prevented by Klotho protein administration [12], although fibrosis has not yet been assessed in such a study.

To summarize, it is generally found that Klotho prevents the development of cardiac fibrosis and the fact that administration of the soluble protein has this effect coupled with the absence of Klotho in cardiomyocytes, lets us conclude that soluble Klotho is likely to directly modulate these effects. Since Klotho is primarily kidney-derived, an implication of these recent studies on cardiac fibrosis is that the renal Klotho supply is integral to the prevention of cardiac fibrosis. Details from the studies in which Klotho overexpression or supplementation has been used in models of cardiac fibrosis, are summarized in Table 2.

2.3. Klotho and fibrosis in other tissues

2.3.1. Arteries

Most studies on the vasculature in Klotho deficiency have focused on the calcification phenotype that plagues the full Klotho knockout. The predominance of this pathological process is likely the reason that it was not recognized until very recently that Klotho$^{+/-}$ mice develop arterial stiffening, characterized by an increase in pulse wave velocity (PWV) and deposition of extracellular matrix in the media [86–88]. Notably, the development of arterial stiffening does not appear to be secondary to the development of hypertension, as arterial stiffening precedes the rise in blood pressure. The increased collagen deposition can be found in the aorta, but not in other large arteries like the carotids and femoral arteries, at least in this age range. The same group then reported that in the high-fat diet model of arterial stiffening, PWV and aortic collagen I protein expression were dramatically increased in mice that were heterozygous for Klotho, indicating that Klotho deficiency exacerbates arterial fibrotic processes as well [87]. No experiments have been performed so far using transgenic mice that overexpress Klotho, using Klotho gene delivery, or administering Klotho protein to assess whether Klotho can prevent the development of arterial stiffening. However, treatment with eplerenone, inhibiting aldosterone signaling [86], SRT1720 treatment, activating SIRT1 [88], and treatment with AMPKα activator AICAR [87] have all been reported to prevent the development of arterial stiffening in Klotho$^{+/-}$ deficient mice. Although it is a controversial topic, there is currently no solid evidence supportive of membrane-bound Klotho expression in arteries [89–91], so the vasculature should, like the heart, be considered a target tissue for soluble Klotho.

Taken together, it has been shown that partial Klotho deficiency in mice both induces and exacerbates fibrotic changes in the aorta, leading to a higher pulse wave velocity. It is yet to be determined whether an increased Klotho level has beneficial effects on arterial fibrotic processes.

2.3.2. Aortic valve

While full Klotho deficiency induces aortic valve calcification [92–94], the aortic valve of Klotho$^{+/-}$ mice has only recently been examined. While there does not appear to be any fibrosis at baseline, a

high-fat diet resulted in marked fibrosis of the aortic valve cusps, including collagen I deposition, primarily on the aortic side [95], indicating that Klotho deficiency may play a role in the pathogenesis of aortic valve stenosis, affecting both aortic valve calcification and fibrosis. It is yet to be investigated whether Klotho overexpression or protein supplementation can counteract aortic valve fibrosis.

2.3.3. Lungs

There are currently two studies in which a relation between Klotho and pulmonary fibrosis has been investigated. Firstly, Kim et al. recently found that although *kl*/+ mice do not exhibit spontaneous pulmonary fibrosis at 11–13 weeks of age, pulmonary fibrosis induced by tracheal instillation of asbestos is exacerbated in *kl*/+ mice as assessed histologically, with an increase in pulmonary collagen content, compared to WT mice [96], fitting with the overall hypothesis that Klotho deficiency renders organs more prone to developing fibrosis. Shin et al. report that ovalbumin-induced pulmonary fibrosis, which was progressive over the course of 4 weeks, was negatively associated with pulmonary Klotho protein expression [97]. However, whether Klotho is expressed in airway epithelium or in lung tissue in general, is not generally accepted [98,99] so this observation warrants further analysis. There are currently no reports on whether Klotho overexpression or supplementation affects pulmonary fibrosis.

2.3.4. Skin

Given that the previous observations have established that Klotho exerts anti-fibrotic effects in *in vivo* models, it is important to address how Klotho affects wound healing. There is limited data on this topic, but a few studies are able to provide us with at least partial answers. Liu et al. were the first to describe that wound healing is impaired in Klotho-deficient mice 4 days after wounding [38]. Another group compared *kl*/*kl*, *kl*/+, and WT mice and found repeatedly that after inflicting a standardized wound, *kl*/*kl* mice displayed slower wound healing [56,100]. On day 7, when *kl*/+ and WT wounds were still 20% open, but *kl*/*kl* wounds were still 80% open, collagen I and III mRNA levels were lower in *kl*/*kl* mice, in line with a lower collagen content on both days 4 and day 7 in these mice [56]. However, Klotho-deficient mice generally develop a thinner dermis with hardly any subcutaneous adipose tissue, compatible with their progeroid phenotype. The delay in wound healing could be attributed to non-intrinsic dermal factors or the influence thereof (such as circulating Klotho?), since grafting of WT skin or *kl*/*kl* skin on WT mice resulted in an undistinguishable wound healing response [100]. Additionally, Klotho expression was not detected in skin, ruling out effects of locally expressed Klotho. Although Klotho-deficient mice apparently do not react to wounding by excessively producing ECM during the process of wound healing, it would be interesting to examine the morphology and composition of healed wounds as it is possible that the resultant scar remodelling and turnover of ECM proteins is impaired, leaving these mice with more fibrosis long-term. It is also possible that other factors that influence wound healing, like angiogenesis, which is impaired in Klotho-deficient mice [101], play a role in delaying wound healing. A final possibility is that there is a mechanistic discrepancy between Klotho effects in "physiological" fibrotic processes as opposed to pathological fibrotic processes. There are currently no studies examining wound healing in Klotho-overexpressing mice or treatment of wounded mice with soluble Klotho.

2.3.5. Underexplored fibrosis models

As fibrosis is a feature of many diseases in many different organs and tissues and Klotho has been firmly established to exert anti-fibrotic

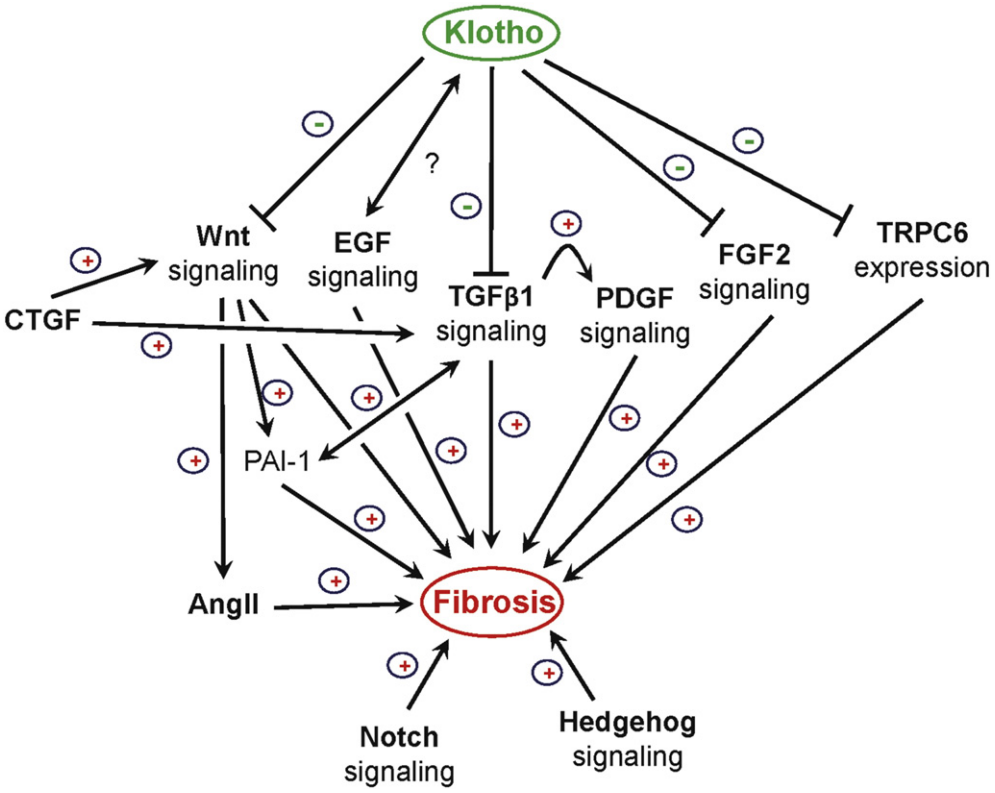

Fig. 2. Fibrosis-related growth factor pathways and their link to Klotho. Many pathways, including CTGF (connective tissue growth factor), TGFβ1 (transforming growth factor β1) signaling, Wnt signaling and downstream plasminogen activator inhibitor 1 (PAI-1) activity, epidermal growth factor (EGF) signaling, platelet-derived growth factor (PDGF) signaling, fibroblast growth factor 2 (FGF2) signaling, renin-angiotensin system activation resulting in high angiotensin II (AngII) levels, transient receptor potential cation channel subfamily C, member 6 (TRPC6) overactivation (downstream of growth hormone signaling), Hedgehog signaling, and Notch signaling have been implicated in fibrosis. Klotho has been shown to inhibit TGFβ1 signaling, Wnt signaling, RAS activation, FGF2 signaling, and TRPC6 expression. The link between Klotho and the EGF pathway is unclear and it is unknown whether a direct link exists between Klotho and PGDF, CTGF, Hedgehog, and Notch signaling. (−): inhibitory effect of Klotho on respective pathway; (+) stimulatory effect of respective pathway on fibrogenesis.

effects *in vivo*, it is of utmost interest to address whether liver fibrosis, intestinal fibrosis, and pulmonary fibrosis (in additional models) can be ameliorated by Klotho treatment. A treatment-related strategy that has also not yet been explored is the possible use of Klotho in preventing radiation-induced fibrosis. If effective, this could lead to the development of a treatment that could be used prior to and/or during radiotherapy, in order to prevent the development of fibrosis. This is an attractive potential application, since radiotherapy is a setting in which the development of fibrosis can be anticipated and preventative treatment could be initiated, unlike the general population setting in which renal, cardiac, or hepatic fibrosis develops insidiously.

3. Molecular mechanisms of action

Having discussed that Klotho is an anti-fibrotic factor *in vivo*, we want to examine next what is known about how Klotho exerts its effects and *via* which pathways. It is rather uncommon that a monogenic disorder, such as Klotho deficiency in knockout mice, produces a phenotype that so strikingly resembles human ageing. It is therefore perhaps not surprising that Klotho deficiency dysregulates a great number of pathways, among which many that are implicated in fibrosis. The known direct molecular interactions between Klotho and target proteins are depicted in Fig. 2.

3.1. Klotho directly inhibits TGFβ1 signaling

Doi et al. first detailed that soluble Klotho directly binds to TGFβRII, and inhibits its affinity for TGFβ1, thereby inhibiting downstream Smad2 phosphorylation, signaling, and αSMA and vimentin expression [37]. They further showed that overexpression of TGFβRII attenuated the inhibitory effect of Klotho on TGFβ1 signaling and constitutively activated TGFβRI abolished it, and that radioactively labelled TGFβ1 is hardly found bound to TGFβRII on the cell-surface after crosslinking in the presence of Klotho. These experiments establish that TGFβRII is the factor in the pathway that Klotho interacts with, both physically and functionally. *In vitro*, soluble Klotho was shown to prevent the expression of TGFβ1-induced fibrogenic genes and proteins [39]. Assessing the biological relevance of this effect *in vivo*, it was shown, in terms of collagen-I and αSMA mRNA expression, that treatment with anti-TGFβ1 antibodies and treatment with Klotho inhibited fibrosis after UUO, but a combination of both treatments did not have an additional effect, suggesting that counteracting TGFβ1 signaling is a mechanism that constitutes at least to a large extent Klotho-mediated prevention of renal fibrosis [37].

3.2. Klotho directly inhibits Wnt signaling

It was demonstrated by Liu et al. using reciprocal immunoprecipitation that circulating Klotho directly binds multiple soluble Wnt molecules, including at least Wnt1, Wnt3, Wnt4 and Wnt5a [38]. Zhou et al. later corroborated that Klotho binds to Wnt1 and Wnt4 [39] while Maltare et al. confirmed the Wnt7a binding capacity of Klotho in kidney lysates [102]. It is thought that the binding of Klotho to Wnt molecules amounts to sequestering them, essentially inhibiting down-stream Wnt signaling. Indeed, luciferase assays have shown that Wnt1 or Wnt3 overexpression-induced reporter activity was diminished dose-dependently after Klotho co-transfection [38,39], but not if constitutively active β-catenin was overexpressed, indicating that Wnt1 is the point of action for Klotho [39]. Klotho overexpression prevented β-catenin activation *in vitro* while repressing expression of its target genes, like PAI-1 and Snail1. *In vivo*, Klotho deficiency leads to overactivation of Wnt signaling, resulting in stem cell senescence and a complex bone phenotype, which could be prevented by Klotho overexpression in this model, but also in a model of constitutive pathological Wnt activation [38]. With regard to fibrosis, loss of Klotho expression in UUO and adriamycin renal fibrosis models was associated with a marked increase in active β-catenin, and Klotho overexpression prevented this change [39,103] as well as the up-regulation of β-catenin target genes and the development of renal fibrosis [39].

PAI-1, as a gene down-stream of Wnt signaling and closely related to TGFβ1, is also an important effector of fibrosis and intricately connected to Klotho. As mentioned, Klotho overexpression will prevent the induction of PAI-1 expression [39]. Conversely, PAI-1 is up-regulated in Klotho deficiency [104] and deletion of PAI-1 in Klotho$^{-/-}$ mice will ameliorate many features of the Klotho deficiency phenotype [105]. While this indicates that PAI-1 is an important factor in the pathogenesis of Klotho deficiency-induced pathologies, fibrosis, however, has not yet been studied in this context.

3.3. Klotho inhibits the expression of renin-angiotensin system genes

Relevant in particular for renal fibrosis is the finding that the genes belonging to the renin-angiotensin system (RAS) are Wnt-induced β-catenin targets [106]. Indeed, Klotho overexpression was shown to inhibit the expression of angiotensinogen, renin, ACE, and AT1 while also inhibiting the development of fibrosis and the deposition of ECM in 5/6th nephrectomy, UUO, and adriamycin nephropathy models [39,66]. In general, it appears to be the case that Klotho decreases angiotensin II expression [66] and prevents angiotensin II-mediated renal damage in a pressure independent fashion [107], while angiotensin II in turn depresses renal Klotho expression [107,108]. Conversely, ACE inhibitors and AT1 receptor antagonists increase Klotho expression, likely by alleviating the angiotensin II-mediated down-regulation of Klotho expression [79,107]. On the other hand, in the heart, it was not found that Klotho prevents cardiac fibrosis induced by angiotensin II. The effect size in this study was very small, as was the induction of fibrosis in this model, but it could signify that Klotho does not have beneficial effects when applied downstream of angiotensin II specifically in the heart. In short, although it is difficult to delineate the effects of different pro-fibrotic pathways that are quite interwoven, antagonizing the RAS is expected to constitute an important contributing anti-fibrotic mechanism for Klotho as well.

3.4. Klotho inhibits FGF2 signaling

An often underappreciated aspect of membrane-bound Klotho functioning as a co-receptor with FGFR1c [35,36], increasing the affinity for FGF23 and potentiating downstream signaling that potentiates phosphaturia and inhibits activation of vitamin D, is the consequent decrease in receptor affinity for FGF2 [41]. As Klotho is progressively down-regulated during disease processes that cause fibrosis, FGF2 signaling is essentially enabled, which in turn drives the development of renal fibrosis. Immunoprecipitation for FGFR1 and immunoblotting for Klotho, FGF2, and FGF23 revealed that FGF2 is co-immunoprecipitated less in the presence of soluble Klotho, while FGF23 is then co-immunoprecipitated more. In terms of competitive binding of FGF23 and FGF2 to Klotho, it is not yet fully clear to what extent this pertains to soluble Klotho, which can bind to FGFR1c but not to potentiate FGF23 signaling [109], and to what extent to membrane-bound Klotho. Soluble Klotho also inhibited FGF2 signaling *in vitro*, suggesting that it is at least partially responsible. Other authors have also found that Klotho overexpression inhibits FGF2 signaling, which could be mediated by either soluble, or membrane-bound Klotho, or both [42]. Highlighting the important role of FGF2 in the pathogenesis of renal fibrosis *in vivo*, it was indeed found that mice that had undergone UUO did not develop as much renal fibrosis if they were knockout for FGF2, while maintaining higher membrane-bound Klotho protein levels than WT UUO mice [41]. The up-regulation of FGF2 during UUO is also blunted by soluble Klotho treatment, potentially indicating a negative feedback regulation, whereas the higher Klotho levels in the absence of FGF2 could reflect the retention of Klotho, rather than up-regulation, although this is yet to be resolved. It is also currently unknown whether depletion of Klotho in

FGF2$^{-/-}$ mice or alternative modulation of FGF2 and/or Klotho levels in combination would support the notion that inhibition of FGF2 signaling is a functionally important effect of Klotho in counteracting fibrosis.

On the other hand, the phosphaturia-enabling effects of FGF23 facilitated by Klotho could also be regarded as a mechanism that counteracts fibrosis, as phosphate has been shown to promote fibrosis, especially in the setting of Klotho deficiency [9].

3.5. Klotho decreases TRPC6 cell surface abundance

The calcium channel Transient receptor potential cation channel, subfamily C, member 6 (TRPC6) is known mostly for its roles in cardiomyocytes and podocytes, in which overactivation is associated with disease. Klotho has been shown to down-regulate TRPC6 expression in the heart and in podocytes, thereby protecting against myocardial hypertrophy [10,11] and podocyte damage that leads to foot process effacement and proteinuria [50], respectively. Since TRPC6$^{-/-}$ mice also display attenuation of renal fibrosis after UUO and no additional benefit from Klotho treatment, there appears to be both a sizeable role for TRPC6 in fibrosis and a common pathway in which Klotho-mediated anti-fibrotic effects involve TRPC6, possibly in renal fibroblasts because of the up-regulation of TRPC6 after UUO in those cells. Unlike the direct enzymatic effects Klotho exerts on various ion channels, the mechanism behind TRPC6 regulation was found to be a PI3K-dependent effect, inhibiting PI3K-mediated exocytosis of TRPC6 channels. There are at least two mechanisms *via* which Klotho is likely to or has been shown to regulate PI3K-mediated TRPC6 cell surface abundance. The first would be inhibition of IGF1 signaling *via* binding to the IGF1 receptor [2,40], thereby also blocking downstream PI3K activation. The second would be inhibition of lipid raft-mediated PI3K and Akt signaling by binding to monosialogangliosides on lipid rafts [110]. RNA *in situ* hybridization has revealed that TRPC6 is particularly up-regulated in interstitial fibroblasts after UUO, suggesting that it may be this cell type that is relevant to the subsequent development of fibrosis. It should also be noted that it may not just concern TRPC6 channels but TRPC6/TRPC3 heteromultimeric channels, if present in renal fibroblasts, since TRPC3$^{-/-}$ and TRPC6$^{-/-}$ mice are protected from UUO-induced fibrosis to an extent similar to TRPC3$^{-/-}$/TRPC6$^{-/-}$ mice, suggesting that both channels may act in the same pathway. It is not known, however, if Klotho affects TRPC3 channel cell surface abundance.

3.6. Other fibrosis-related pathways

A great number of pathways has been implicated in fibrosis, some of which have been linked to Klotho, albeit in a more indirect manner than the aforementioned major fibrosis-related pathways. For instance, an important pro-fibrotic pathway in renal fibrosis is epidermal growth factor (EGF) signaling. Klotho probably plays a role in EGF signaling, since Klotho deficiency leads to a decrease in EGF expression, at least in the lung [111] and EGF has been shown to promote Klotho transcription [112]. Furthermore, Klotho has been shown not to bind to EGFR [37]. In short, the link between Klotho and EGF signaling is not yet properly characterized and it is yet to be determined whether there is any relevance to fibrosis. Similarly, connective tissue growth factor (CTGF) signaling is known to be involved in renal fibrosis, generally promoting TGFβ1 and Wnt signaling. It is unknown whether Klotho affects CTGF signaling, although Klotho did not co-immunoprecipitate with CTGF receptor LRP6 [37]. Another important pathway involved in fibrosis is platelet-derived growth factor (PDGF) signaling, but it is unknown whether Klotho affects this pathway, other than that it does not bind to PDGFRα [37]. A pathway in which is Klotho is known to be involved is mammalian target of rapamycin (mTOR) signaling, which is the case at least because mTOR acts downstream of IGF1R. As expected, Klotho deficiency leads to increased mTOR signaling [49]. However, mTOR also appears to act somewhere upstream of Klotho, since mTOR has been shown to inhibit vascular calcification in CKD models, but not in Klotho$^{-/-}$ mice, indicating that mTOR affects this process *via* Klotho [113]. Sustained Notch and Hedgehog signaling have also been implicated in renal fibrosis and given their interactions with the Wnt and TGFβ1 pathways, may be altered in response to Klotho as well. All in all, a number of pathways involved in fibrosis has been linked to Klotho (see Fig. 2), but the molecular mechanisms are not yet completely understood. In addition, a number of pathways is not known to be associated with Klotho, but given their involvement in the same process of fibrosis, it may be worthwhile to address whether these pathways intersect, or act independently.

4. Klotho and fibroblasts

Having assessed the most important pathways Klotho appears to be involved in, it may be of interest to look more broadly at the effects Klotho has on fibroblasts. The first question that has to be addressed is whether Klotho is expressed by fibroblasts themselves. Data on this topic are conflicting. First of all, Azuma et al. did not detect any Klotho mRNA or protein in renal fibroblasts [114] and Pásztói et al. detected a very low level of Klotho mRNA in synovial fibroblasts [115]. Similarly, in renal interstitial fibroblasts, Huang et al. describe low Klotho mRNA and protein levels, which increased, however, upon high glucose stimulation [116]. More recently, Lee et al. report that Klotho mRNA expression is not found in native porcine fetal fibroblasts [117]. On the other hand, Liang et al. report Klotho immunostaining in tenocytes [118] and multiple authors detect immunoreactivity in human skin fibroblasts [119,120], MRC5 cells [121], and lung fibroblast cell line WI-38 [122]. It is difficult to place these findings in a proper context. While Azuma et al. used both renal cells as a positive control and antibody KM2076 to detect Klotho [114], which is the most frequently used and best-validated antibody for human Klotho, Liang et al. do not indicate which antibody they used [118], De Oliveira et al. do not indicate what size their detected protein is [121], and three other studies describe smaller proteins of 64 and 116 kDa [119,120,122]. Markiewicz et al. even report Klotho mRNA expression about two-fold higher than β2-microglobulin mRNA expression [120]. While it is certainly possible that Klotho is expressed in some form or at a low level in fibroblasts, or in some specialized types of fibroblast-like cells but not in others, there is currently no solid evidence of fibroblast Klotho expression. In any case, with regard to renal fibroblasts, it is difficult to envision a role for fibroblast Klotho in inhibiting renal fibrosis, also because the Klotho expression pattern in the kidney has been studied extensively and no authors have ever reported positive staining in interstitial fibroblasts (see Fig. 1B). Future studies should be performed to elucidate this issue, ideally aided by validated antibodies, *in situ* hybridization, and knockout tissue.

Effects of Klotho itself on fibroblasts have also been investigated only sporadically. Markiewicz et al. describe that Klotho treatment inhibited dermal fibroblast migration, while silencing of Klotho promoted it [120]. De Oliveira et al. describe that Klotho knockdown inhibited proliferation of fibroblasts, which appeared to be due to an increase in p53-mediated senescence [121]. Klotho overexpression was found to decrease IL-6 production in mouse embryonic fibroblasts isolated from both WT mice and *kl*/*kl* mice [122]. The fibroblasts from *kl*/*kl* mice displayed an exaggerated IL-6 production compared to the WT fibroblasts, although Klotho overexpression essentially brought the IL-6 level back to WT levels. Furthermore, IL-6 production in *kl*/*kl* mouse embryonic fibroblasts was decreased after silencing of RIG-I. It was coined that a direct interaction between RIG-I and an intracellularly expressed KL1 domain in endothelial cells prevents RIG-I-mediated inflammation, although it was not further substantiated whether this is the case in fibroblasts and whether an intracellular Klotho protein physiologically performs this function [122]. In renal interstitial fibroblasts, Klotho was found to revert the high-glucose induced expression of TGFβRII, at least relative to TGFβRI, preventing downstream Smad2/3 phosphorylation [116]. High-glucose-induced p38 and ERK1/2 phosphorylation

were inhibited as well, and ultimately this resulted in a decrease in high-glucose-induced cell hypertrophy and fibronectin expression. In porcine fibroblasts, transgenic Klotho overexpression increased IGF1 mRNA expression, as well as expression of anti-oxidant defense factors FOXO1, Mn-SOD, and catalase [117]. Expression of p53 and p16, genes encoding proteins that promote cellular senescence, caspase 3, and DNA methyltransferases was decreased. Interestingly, using the nuclei of these transgenic fibroblasts increased blastocyst formation, possibly pointing towards a better cellular health resulting in improved survival.

In general, however, not many studies have addressed the effects of Klotho on fibroblasts in terms of proliferation, migration, differentiation, and synthesis of extracellular matrix. A study by Liu et al. found differential effects depending on whether cardiac fibroblasts were treated with the 130 kDa Klotho protein, leading to higher collagen-I and αSMA expression, ERK phosphorylation, and proliferation, or whether cells were treated with KL1, leading to a decrease in proliferation and collagen-I production. While it is possible that different Klotho proteins exert different effects, Hu et al. investigated neonatal cardiac fibroblasts and did find that Klotho treatment (the 130 kDa protein) inhibited TGFβ1-induced CTGF expression, Ang-II-induced collagen-I expression, and phosphate-induced expression of both CTGF and collagen-I [9]. Although Smad2/3 phosphorylation was not found to be induced or inhibited by any treatment in these cells, Klotho did prevent ERK phosphorylation induced by either TGFβ1, AngII, or phosphate. Taken together, these data indicate that Klotho may protect fibroblasts from senescence and may inhibit the synthesis of extracellular matrix, although some conflicting data complicate the overall picture. As it appears that Klotho can inhibit the pathological de-differentiation of vascular smooth muscle cells [20], and prevent epithelial-to-mesenchymal transition of renal HK-2 cells [41], a central question for fibroblast research remains what the effect of Klotho is on fibroblast differentiation to myofibroblasts, as well as on subsequent ECM synthesis.

5. Klotho in cancer

When it was found that Klotho extends lifespan at least in part by inhibiting IGF1/insulin signaling, it was quickly hypothesized that Klotho may have anti-tumor effects [2,123]. Not long after, experiments were performed that supported anti-tumor effects by Klotho through targeting of IGF1/insulin signaling as well as other oncogenic pathways [40]. As many of the pathways addressed in the context of fibrosis are also relevant in cancer biology, it is of particular interest to discuss the effects of Klotho on tumors in this review. It should be noted that Klotho$^{-/-}$ mice are not known to develop tumors, although a potential increased propensity to develop cancer might be obfuscated by their short lifespan. Notably, Klotho$^{+/-}$ mice are also not known to develop tumors spontaneously despite their near-normal lifespan. An interesting relationship to mention is the one between p16^{Ink4a}, a well-established tumor suppressor that induces cellular senescence, and Klotho, which is down-regulated by the former [82].

5.1. Klotho expression in tumors

It should be noted that Klotho expression is generally considered to be extremely low in most tissues (aside from the kidney, parathyroid gland, and choroid plexus), so any biological relevance of any further down-regulation, if established, may indicate that our current views on Klotho expression levels require some thorough evaluation. On the other hand, it should be noted that different anti-Klotho antibodies are known to yield discrepant results. This is likely due to unspecific antigen binding, which may produce both false-positive and false-negative results [98]. As a general rule, though, the Klotho promoter is hypermethylated in tumor tissue and Klotho expression is consequently decreased. This was first demonstrated by Lee et al. who showed that the Klotho promoter is frequently hypermethylated in cervical carcinoma [124]. This was later also shown to be the case in colorectal carcinoma [125–127], gastric carcinoma [128,129], mamma carcinoma [130–132], hepatocellular carcinoma [133], pancreatic adenocarcinoma [134], and even chordoma [135]. Klotho expression is also silenced by histone deacetylation in various tumors [124]. As a result, Klotho gene and protein expression have been shown to be decreased in esophageal carcinoma [136], gastric carcinoma [128,137], pancreatic carcinoma [42,134], breast cancer [40,130,132], colorectal carcinoma [125,138], cervical carcinoma [139,140], hepatocellular carcinoma [133,141], renal cell carcinoma [142], ovarian carcinoma [143,144], glial tumors [145], urothelial carcinoma [146], oral squamous cell carcinoma [147], and diffuse large B cell lymphoma (DLBCL) [148]. In contrast, Klotho expression may be increased in multiple myeloma [149]. Taken together, the available data indicate that Klotho is nearly universally silenced upon oncogenesis. Of note is the observation that it was generally found to be the case that residual Klotho expression, however little, was still associated with a better outcome [128,133,134,136–138,141–143,148,150–152].

5.2. Klotho effects on cancer cells in vitro and in vivo

As stated, Klotho inhibits many pathways involved in carcinogenesis, including IGF1R (with downstream PI3K, Akt, and mTOR signaling), Wnt/β-catenin signaling, FGF2 signaling (generally affecting ERK1/2 signaling), and TGFβ1 and downstream Smad2/3 signaling. Generally, *in vitro* anti-tumor effects were found to include the induction of apoptosis [125,128,129,141,145,148,153–158], inhibition of proliferation [40,42,43,124,125,128,138,141,143–145,148,153–159], induction of autophagy [129,159], inhibition of autophagy [155], and inhibition of migration [37,138,139,142,154,159,160]. Klotho effects were found to be mediated by inhibition of activation of IGF1R (and/or downstream PI3K and Akt signaling) [40,42,43,129,138,142,144,148,153,157,159], ERK1/2 signaling [42,128], Wnt/β-catenin signaling [124,139,141,154,160,161], and TGFβ1 signaling [37]. Notably, the EGF pathway has not been found to be modulated by Klotho [40], although Klotho-deficient mice are known to have low EGF levels [111]. Interestingly, Klotho treatment appears to interact favourably with cytostatics in resistant cell lines [42,144,155,157].

In vivo, it was shown that Klotho significantly inhibited tumor growth and/or improved survival in athymic mice xenotransplanted with lung cancer [37], pancreatic carcinoma [42], colorectal carcinoma [162], breast cancer [43], hepatocellular carcinoma [141], ovarian carcinoma [143], melanoma [161], and diffuse large B cell lymphoma [148]. These studies have used different approaches to Klotho treatment. While the pancreatic carcinoma, breast cancer, hepatocellular carcinoma, and melanoma experiments were performed with recombinant soluble Klotho treatment, Klotho was overexpressed in the models of colorectal and ovarian carcinoma. The DLBCL experiments did both and the lung cancer experiments by Doi et al. also employed both approaches, in addition to injecting lung cancer cells into WT and Klotho-overexpressing mice, all of which resulted in fewer metastases after exposure to higher Klotho levels, likely through an effect on the TGFβ1 pathway [37]. Additionally, an *in vivo* study in which Klotho was down-regulated in melanoma cells using a short hairpin RNA showed that mortality and tumor growth were increased [152]. Finally, a similar approach using short hairpin RNAs against Klotho in cisplatin-resistant lung cancer cells also lead to an increase in tumor volume, further establishing a role for Klotho as a tumor suppressor [157]. The available data indicate that Klotho acts as a universal tumor suppressor and that there may be a role for Klotho in the treatment of cancers.

6. Strategies for Klotho treatment

Currently, there are no Klotho-based treatments available, although a number of commonly used compounds do either directly up-regulate Klotho *in vitro*, like PPARγ agonists [73,163], vitamin D [164], testosterone [165], and resveratrol [166], or otherwise appear to up-regulate or

at least de-repress down-regulation of Klotho *in vivo*, like ACE inhibitors/AT1R blockers [79,167], statins [64], and *N*-acetylcysteine [63]. Establishing higher renal Klotho expression levels or systemic soluble Klotho levels could therefore be achieved by treatment with these compounds. However, in the presence of factors that actively down-regulate Klotho, like uremic toxins [69], or in case of advanced renal disease, in which tubular cells may no longer possess the ability to express Klotho, the effect of up-regulating endogenous Klotho levels may be mild to absent. It is therefore interesting, given the established *in vivo* effects of soluble Klotho protein and for the potential treatment of ESRD patients, to also explore the possibilities for exogenous soluble Klotho treatment.

A few things should be noted on Klotho-based treatments. First of all, if the goal is to maintain Klotho levels at a physiological level, which is expected to confer a certain degree of protection compared to disease states in which Klotho is either locally or systemically down-regulated, then there are currently no indications that such a treatment would be expected to cause undesirable or adverse side effects. Raising endogenous Klotho levels to about twice the normal level, as present in the transgenic Klotho-overexpressing mouse, also does not appear to induce unwanted side effects. Given the general beneficial effects of high circulating Klotho levels on the entire organism, it could be argued that the full protein with all of its functions intact would be suitable for

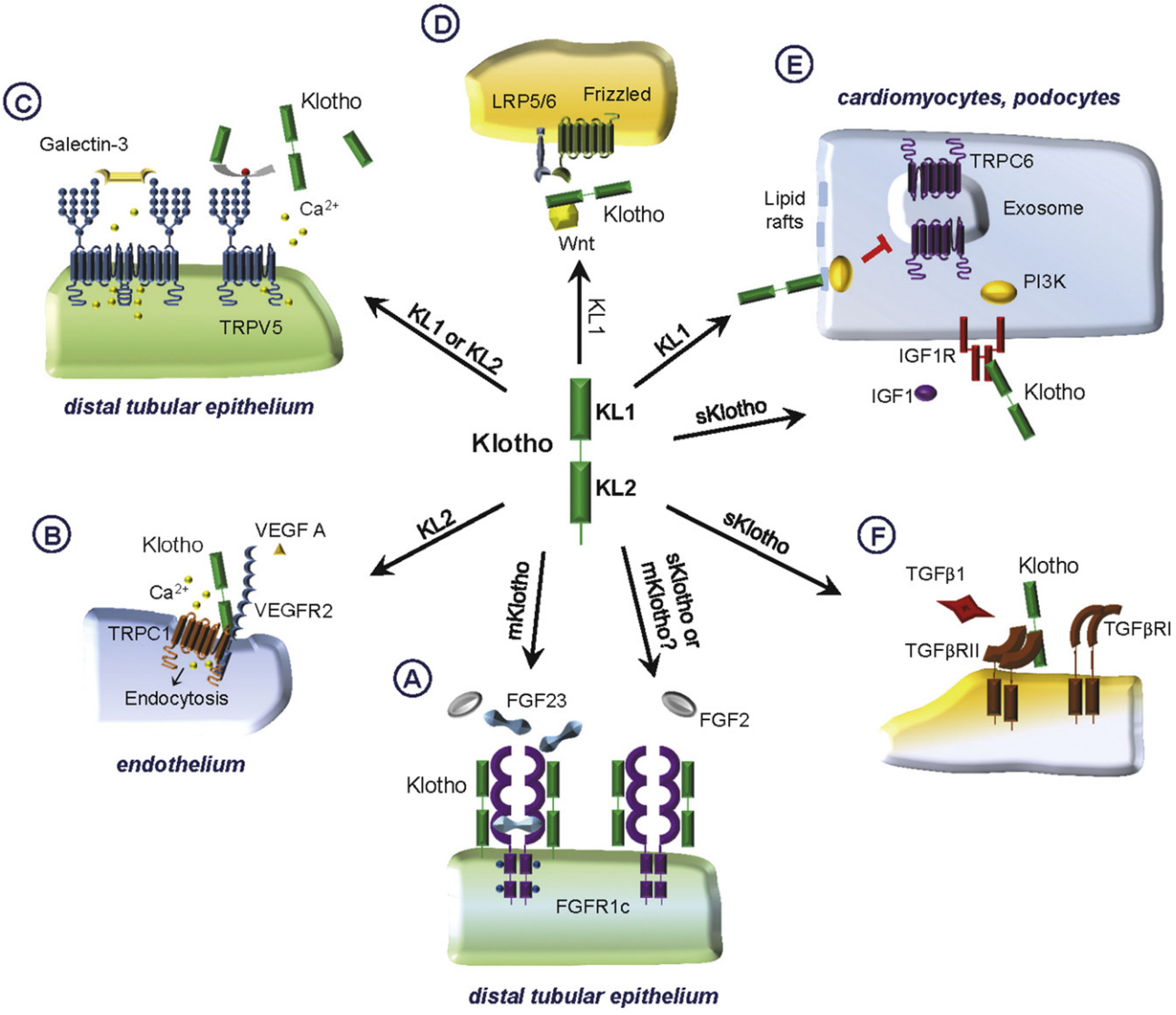

Fig. 3. Direct Klotho-mediated molecular mechanisms of action. Klotho (depicted in the centre) directly interacts with other pathways. Clockwise from the bottom panel: (A) on renal distal tubule epithelial cells, membrane-bound Klotho binds to fibroblast growth factor receptor 1c (FGFR1c) to form a ternary complex with FGF23, facilitating phosphaturia in the proximal tubule. Binding of Klotho to FGFR1c reduces the affinity for FGF2, inhibiting FGF2 signaling. (B) On endothelial cells, the KL2 domain binds to Ig6 and 7 from vascular endothelial growth factor receptor 2 (VEGFR2) and to the fifth loop of transient receptor potential cation channel, subfamily C, member 1 (TRPC1), forming a complex that is taken up by endocytosis upon stimulation by VEGF, protecting the cell from calcium-induced μ-calpain overactivation. (C) On distal tubule epithelium in the kidney, Klotho enzymatically modifies the N-linked glycan on transient receptor potential cation channel, subfamily V, member 5 (TRPV5), which allows stabilization of TRPV5 molecules by galectin-3 on the cell membrane, promoting calcium reabsorption from the urine. Klotho acts as a sialidase or β-glucoronidase on other ion channels and transporters as well, including sodium and phosphate transporter NaPi2a and potassium channel ROMK1. (D) The KL1 domain is capable of sequestering soluble Wnt molecules, preventing Wnt signaling. (E) The KL1 domain also inhibits PI3K signaling, either by inhibiting insulin-like growth factor 1 (IGF1) signaling by binding to IGFR1, or by binding monosialogangliosides on lipid rafts in the cellular membrane. Inhibition of downstream PI3K and Akt signaling will prevent exocytosis of transient receptor potential cation channel, subfamily C, member 6 (TRPC6) on cardiomyocytes and podocytes. (F) Soluble Klotho binds to transforming growth factor β1 receptor II (TGFβRII), reducing the affinity for TGFβ1 and preventing the heterodimerization of TGFβRII and TGFβRI, thereby inhibiting downstream Smad2/3 signaling. Abbreviations: FGF: fibroblast growth factor; FGFR: fibroblast growth factor receptor; IGF1: insulin-like growth factor 1; IGFR: insulin-like growth factor receptor; LRP6: low-density lipoprotein receptor-related protein 6; mKlotho: membrane-bound Klotho; PI3K: phospho-inositide 3-kinase; sKlotho: soluble Klotho; TGFβ1: transforming growth factor β1; TGFβR – transforming growth factor β receptor; TRPC1: transient receptor potential cation channel subfamily C, member 1; TRPC6: transient receptor potential cation channel, subfamily C, member 6; TRPV5: transient receptor potential cation channel, subfamily V, member 5; VEGF: vascular endothelial growth factor; VEGFR2: vascular endothelial growth factor receptor 1; Wnt: wingless-related integration site.

treatment. However, if Klotho levels are increased to an even higher level, a number of side effects can be expected, including hypotension, hypophosphatemia [168,169], hypocalcemia [168], and insulin resistance that may start to affect glucose levels [2]. Therefore, it may be worthwhile to discuss whether the different Klotho effects can be delineated, so that side effects with regard to phosphate homeostasis can be prevented while still affecting, for example, calcium homeostasis or Wnt signaling, or so that a higher level of Klotho with specific biological activity in a certain pathway can be reached without the occurrence of side effects. Finally, we will discuss the targeted treatment strategies that have been employed experimentally using Klotho, which could enable us to reach an even higher Klotho level locally, if that is biologically desirable and effective. These approaches could be coupled with modified Klotho protein treatment acting only on certain pathways, if the biological effect in a certain tissue can then be maximized [1,25,26] [27–29] [30–32] [33,34] [27,28]..

6.1. Structure-function analyses

As Klotho effects can conceivably be exerted by membrane-bound Klotho, full-length soluble Klotho, or the individual KL1 or KL2 domains, a few groups have tried to pinpoint which Klotho protein or which domain of the Klotho protein is necessary in exerting different functions. FGF23 signaling, for instance, has only been found to occur in the presence of membrane-bound Klotho [109], which is therefore likely the protein that affects phosphate homeostasis the most, although soluble Klotho is also capable of inducing phosphaturia independently of FGF23 signaling, by interacting with NaPi2a directly after transcytosis through proximal tubular epithelium [170,171]. Mutagenesis studies indicate that the KL2 domain of soluble Klotho is necessary for binding to TRPC1 and VEGFR2 on endothelial cells, enabling VEGF-mediated endocytocis of TRPC1 and preventing calcium-dependent μ-calpain overactivation [172]. Either KL1, KL2, or the full soluble protein is capable of enzymatically modifying sugar moieties on TRPV5 [173] or NaPi2a [171], as corroborated by blocking the KL1 or KL2 domain using KL1- or KL2-specific antibodies. Further mutagenesis studies have indicated that the KL1 domain is sufficient for blocking Wnt signaling [38] as well as for inhibition of the PI3K/Akt signaling pathway by binding of Klotho to lipid raft-associated α2,3-siallyllactose, for which Arg148, His246, and the ^{465}EWHR^{468} motif in the KL1 domain are likely particularly important [174]. KL1 is also sufficient for the exertion of anti-tumor effects, attributed to inhibition of IGF1 signaling [42,43]. As demonstrated specifically by Abramovitz et al., treatment with high levels of full-length Klotho or KL1 protein both resulted in a reduction in tumor size, but KL1 did not affect phosphate levels, indicating that it may indeed be possible to extricate a given Klotho function from the rest of the functions and that this can have biologically relevant effects. If such advancements are to be explored in therapeutic strategies, it will be necessary to gain a better understanding of the exact molecular mechanisms through which Klotho affects other pathways, both the molecular interactions that are currently known and any that are currently unknown. Elucidation of the tertiary structure of Klotho proteins using X ray crystallography, *in silico* analyses, such as recently performed by Mirza et al. for Klotho/Wnt interactions [175], and mutagenesis studies will be crucial in this effort. It may be possible, though, to develop a Klotho-derived peptide or protein that exhibits all of the anti-fibrotic effects of Klotho and can be administered at a high dose if pathways that would have resulted in side effects remain unaffected. The known direct interactions between Klotho, or specific domains of the Klotho protein, and target proteins are summarized in Fig. 3.

6.2. Klotho treatment strategies

Devising a strategy to deliver Klotho specifically to certain cells or a certain tissue, thereby effectively allowing for higher dosing locally and preventing systemic side effects, is an attractive option. A couple of divergent approaches have been tried – although none in a model of fibrosis. For example, Varshney et al. overexpressed the KL1 domain in stem cells and injected these cells in monocrotaline-treated rats that develop pulmonary hypertension [176]. While the stem cells themselves (in the control condition) did not have an effect on the development of pulmonary hypertension, they did home to the lung and engraft. Klotho overexpression in these cells resulted in a marked reduction in pulmonary vascular dysfunction, arterial remodelling, and cardiac parameters of right ventricular overload. A different approach may be to overexpress Klotho in a specific cell type, as Lin et al. have done in pancreatic β cells, using the β cell-specific pre-pro-insulin II promoter, which inhibited the development of diabetes [23,24]. Such an approach, targeting myofibroblasts, may also result in higher local Klotho levels and may then similarly inhibit the development of fibrosis.

Klotho protein administration in the experimental setting is typically done intraperitoneally. Hu et al. recently showed that treating CKD mice with Klotho protein *via* osmotic mini-pumps, which were changed monthly, still resulted in marked amelioration of CKD and related pathologies [8]. Of note is the observation that recombinant Klotho protein is apparently stable enough, or its efficacy potent enough, to have a therapeutically relevant effect even if renewed only monthly. This finding opens up a lot of treatment possibilities with regard to slow release depots of recombinant Klotho protein that can be injected or implanted. A similar study, by Cheng et al., employed Klotho-loaded chistosan/β-cyclodextrin nanoparticles to coat decellularized arterial grafts and found that 9/10 Klotho-treated grafts remained patent after 6 months, in addition to having developed a normal arterial histology, endothelial lining, and blood flow rate [177]. Comparatively, 9/10 control grafts were no longer patent after 6 months. This study illustrates both the substantial clinical potential of a Klotho-based treatment and the tolerance of a microenvironment to high levels of Klotho. All in all, there might conceivably be a role for Klotho delivery using various strategies and in various situations as these studies may just be setting the stage for investigations to come in this field.

A number of technical aspects is yet to be addressed, before clinical testing of Klotho treatment can commence. First of all, there is an urgent need in the field for a validated ELISA [46,178–181]. A focus of the development of an ELISA would also have to be which Klotho protein should be and are detected, since the current ELISA assays are thought to detect only full-length soluble Klotho. Second of all, it has proven challenging to produce recombinant Klotho with consistent biological effects. However, if these issues are overcome, it could be envisioned for recombinant Klotho treatment to be incorporated into clinical practice as has been the case with other recombinant proteins. For instance, recombinant Klotho treatment could even be combined with daily subcutaneous insulin injections, aiming to protect against diabetic nephropathy, or with regular dialysis-related erythropoietin treatments, aiming to relieve the effects of systemic Klotho deficiency in ESRD. As discussed, the benefits and clinical applications of designed Klotho-based treatments may even stretch further, once scientific progress enables us to devise them for specific purposes.

7. Conclusion

Klotho is a kidney-derived endogenous anti-fibrotic agent. Klotho deficiency results in, or at least exacerbates, the development of fibrosis in the kidney, in the heart, in arteries, in the aortic valve, in the lungs, and negatively affects wound healing. Conversely, Klotho overexpression protects against the development of fibrosis in kidney and heart. These effects are mediated by soluble Klotho, which directly inhibits TGFβ1, Wnt, and FGF2 signaling. Inhibition of PI3K-induced exocytocis of TRPC6 may also be an important factor in preventing renal fibrosis. Interacting with these pathways renders Klotho a tumor suppressor as well. The KL1 domain appears to be integral to most anti-fibrotic functions. Further study on the molecular mechanisms of Klotho-mediated actions will enable designed, possibly KL1-based treatment strategies

that target certain pathways but not others. Klotho-based treatment strategies are a very promising possibility, both for fibrosis and cancer.

Competing financial interests

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